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Rapid Differentiation of Mixed Influenza A/H1N1 Virus Infections with Seasonal and Pandemic Variants by Multitemperature Single-Stranded Conformational Polymorphism Analysis▽

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Mixed infections of a single host with different variants of influenza A virus are the main source of reassortants which may have unpredictable properties when they establish themselves in the human population. In this report we describe a method for rapid detection of mixed influenza virus infections with the seasonal A/H1N1 human strain and the pandemic A/H1N1/v strain which emerged in 2009 in Mexico and the United States. The influenza virus A/H1N1 variants were characterized by the multitemperature single-stranded conformational polymorphism (MSSCP) method. The MSSCP gel patterns of hemagglutinin gene fragments of pandemic A/H1N1/v and different seasonal A/H1N1 strains were easily distinguishable 2 h after completion of reverse transcription-PCR (RT-PCR). Using the MSSCP-based genotyping approach, coinfections with seasonal and pandemic variants of the A/H1N1 subtype were identified in 4 out of 23 primary samples obtained from patients that presented with influenza-like symptoms to hospitals across Poland during the 2009-2010 epidemic season. Pandemic influenza virus strain presence was confirmed in all these primary samples by real-time RT-PCR. The sensitivity level of the MSSCP-based minor genetic variant detection was 0.1%, as determined on a mixture of DNA fragments obtained from amplification of the hemagglutinin gene of seasonal and pandemic strains. The high sensitivity of the method suggests its applicability for characterization of new viral variants long before they become dominant.

Influenza A viruses are divided into 16 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes. The genetic material of the virus is composed of eight negative-stranded RNA segments, with each discrete segment coding for one or two proteins. This genetic property of the virus allows genes to be exchanged between two (or more) genetic variants separately from the rest of the genome. The process, known as reassortment, can lead to abrupt changes in the genetic pool of the virus and may contribute to the emergence of new viral subtypes with pandemic potential (5).

In every epidemic season, different types/variants of influenza viruses cocirculate in the human population; thus, mixed infections with more than one type/variant of the virus should be considered and monitored. The most serious consequence of coinfection is the possibility of viral reassortment. Detailed phylogenetic analyses provided by Nelson and colleagues suggest that segmental reassortments have played an important role in the evolution of influenza A viruses (10). The unusually severe outbreaks of influenza in 1947 and 1950-1951 were probably due to mixed infections which resulted in several intrasubtype reassortments, though the serotype of the outcoming virus was not changed.

The A/H1N1/v strain responsible for the first pandemic of the 21st century turned out to be relatively mild compared with influenza viruses responsible for other pandemics of the past or even with the seasonal strains (13). However, such a situation may be transient, and there are concerns that the virus may reassort to a more pathogenic variant. Moreover, the cocirculation of pandemic and seasonal virus and the common occurrence of oseltamivir resistance in seasonal A/H1N1 strains could lead to the establishment of an oseltamivir-resistant pandemic strain in the patient (1, 2, 6). Therefore, it is of vital importance to monitor mixed seasonal/pandemic influenza virus infections to provide data for more accurate epidemiological investigation.

Although there are confirmed cases of cocirculation of pandemic A/H1N1/v and seasonal A/H1N1 and A/H3N2 viruses during the epidemic season of 2009-2010, only a few reports show mixed infections in patients (4, 6, 8, 9). Thus, the aim of our study was to develop and evaluate a new molecular method for rapid identification and discrimination of genetic variants of influenza A/H1N1 virus, including the detection of mixed infections in a single patient. The minor genetic variant detection method developed and applied in this work is based on the known multitemperature single-stranded conformational polymorphism (MSSCP) analytical and preparative capabilities (7, 11). We evaluated the MSSCP approach on 2009 pandemic

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TABLE 1. Geographical location, travel history, and contact with already confirmed A/H1N1/v cases for patients from whom specimens for analysis were collected

Lane no. on Fig. 2	Sample no.	Geographical location of patient[a]	Contact with a confirmed A/H1N1/v-infected patient	Time between onset of symptoms and specimen collection (days)	Sex[b]	Age (yr)
1	110	USA	—[c]	0	M	43
2	193	Canada	—	3	M	3
3	195	—	Patient 193	0	M	6
4	244	Canada	—	1	M	13
5	253	UK	—	1	F	29
6	256	—	Patient 244	1	M	18
7	272	Canada	Patient 244	3	F	11
8	360	Spain		3	F	28
9	383	—	Patient who returned from England	Without symptoms	M	28
10	384	—	Patient who returned from England	Without symptoms	F	3
11	415	Spain	—	3	F	18
12	462	Holland	—	1	F	15
13	667	India	—	1	F	26
14	682	Mexico and USA	—	3	M	49
15	692	Northeast Poland	—	2	F	7
16	702	Central Poland	—	5	M	11
17	779	Northwest Poland		2	F	15
18	751	Central Poland	—	—[d]	M	47
19	723	East Poland	—	1	M	20
20	911	Southwest Poland	—	1	M	7
21	837	Central Poland	—	—	M	70
22	888	Southeast Poland	—	2	F	47
23	736	North Poland	—	—	F	21

[a] The country visited by the subject or the region of Poland where the subject lived. Those who visited another country returned during the week before the onset of influenza-like illness symptoms.
[b] M, male; F, female.
[c] —, no data available.
[d] Postmortem.

influenza A virus (H1N1)-positive specimens collected from flu-diagnosed patients in Poland and confirmed to be pandemic influenza A virus (H1N1) by real-time reverse transcription-PCR (RT-PCR) during the epidemic season of 2009-2010.

MATERIALS AND METHODS

Virus strains and specimens. The present study included 8 reference strains of seasonal influenza virus A/H1N1 (A/Brisbane/59/07, A/Solomon Islands/03/06, A/New Caledonia/20/99, A/Fukushima/141/06, A/Fukushima/97/06, A/Hong Kong/2652/06, A/St. Petersburg/10/07, A/Taiwan/01/86), pandemic influenza viruses A/H1N1/v [A/Mexico/4486/09, A/England/195/09, A/Gdansk/037/2009 (H1N1), A/Gdansk/036/2009 (H1N1)], and 23 respiratory specimens (nasal and throat swabs) obtained from patients with laboratory-confirmed cases of infection with A/H1N1/v. Information about the patients' geographic location across Poland and their travel histories are summarized in Table 1. Respiratory specimens were collected and transported in sterile physiological saline at 4°C within 24 h. RNA was immediately isolated, or specimens were stored at 4°C for several hours until they were processed. All clinical specimens tested positive for influenza A virus by classical RT-PCR and for pandemic A/H1N1/v by real-time RT-PCR (data not shown).

RNA extraction. Viral RNA was extracted from 140-μl samples using a QIAamp RNA viral minikit (Qiagen, Germany), according to the manufacturer's instructions. RNA was eluted in 50 μl of elution buffer and stored at −80°C.

RT-PCR. To be applicable for MSSCP analyses of H1N1 variants, the primers must fulfill two conditions: first, they have to anneal well to the complementary region of the HA gene of all examined strains, and second, the HA sequences amplified with this set of primers should show high variability in A/H1N1 viruses. In accordance with the first condition, the reverse transcription followed by DNA amplification in the RT-PCR yielded fragments of about 180 bp for all reference seasonal A/H1N1 strains and pandemic A/H1N1/v influenza viruses. Primers specific for the HA gene of both pandemic A/H1N1/v and seasonal A/H1N1 strain were designed: H1msscp1 (5′-AGTAACACACTCTGT-3′) and H1msscp2 (5′-ACAATGTAGGACCATGA-3′). The primers were synthesized by IBB PAN (Warsaw, Poland). RT-PCR was performed in a 25-μl reaction mixture volume with a Transcriptor one-step RT-PCR kit (Roche Diagnostics, Switzerland), 0.4 μM each primer, and 5 μl of RNA solution. The assay was performed in a Veriti 96-well thermal cycler (Applied Biosystems Inc.) as follows: a single cycle of reverse transcription for 30 min at 50°C and 7 min at 94°C for reverse transcriptase inactivation and initial denaturation and then 45 cycles of denaturation at 94°C for 10 s, annealing at 46°C for 30 s, and extension at 68°C for 35 s. After the last cycle, the reaction was completed by a final extension at 68°C for 7 min.

MSSCP-based minor variant enrichment procedure. The RT-PCR products were analyzed by the MSSCP method at a strictly controlled (to ±0.2°C) gel temperature in dedicated equipment, a DNAPointer system (BioVectis, Warsaw, Poland), as described by Kaczanowski et al. (7). The RT-PCR products were heat denatured and resolved as single-stranded DNA (ssDNA) conformers on a 9% polyacrylamide gel under native conditions (TBE [Tris-borate-EDTA] buffer) at three different temperatures during one run. Subsequently, DNA bands were visualized by silver nitrate staining (SilverStain DNA kit; BioVectis, Warsaw, Poland). Fragments of the MSSCP gel containing bands of interest were cut out, and ssDNA was eluted and reamplified using the primers and PCR conditions described above. For subsequent DNA Sanger sequencing (12), a 1/10 volume of PCR products was used (3730xl DNA analyzer; Applied Biosystems, Carlsbad, CA). To estimate the sensitivity of the minor genetic variant detection procedure based on the MSSCP separation, RT-PCR products of the hemagglutinin gene region from seasonal and pandemic variants were mixed at proportions of 50% and 50% down to 0.1% and 99.9% and then analyzed by the MSSCP method.

RESULTS

In this retrospective study, 23 respiratory specimens were selected from numerous samples collected from patients with flu symptoms in Poland during the 2009-2010 influenza season. From all the samples, the hemagglutinin gene fragments between nucleotides 125 and 302 were amplified by the RT-PCR

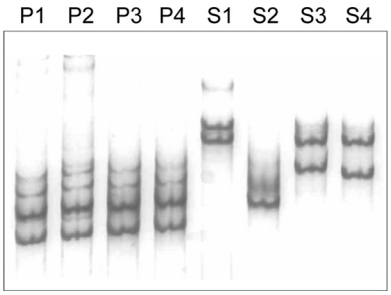

FIG. 1. MSSCP genotyping method differentiates pandemic and seasonal strains of influenza virus A/H1N1. Products of hemagglutinin gene amplification obtained for pandemic A/H1N1/v and reference seasonal strains of influenza virus A/H1N1 were denatured, and ssDNA was separated in a 9% polyacrylamide gel using the MSSCP method under optimal electrophoretic conditions. DNA bands were visualized with silver stain. Strains are indicated as follows: pandemic (P) strains were A/Mexico/4486/09 (lane P1), A/England/195/09 (lane P2), A/Gdansk/037/2009(H1N1) (lane P3), and A/Gdansk/036/2009 (H1N1) (lane P4). Reference seasonal (S) strains were A/Brisbane/59/2007 (lane S1) A/Hong Kong/2652/2006 (lane S20), A/New Caledonia/20/1999 (lane S3), and A/Solomon Islands/3/2006 (lane S4).

method as described in Materials and Methods. This region corresponds to the sequence of the influenza virus HA1 polypeptide, which starts 26 amino acids after the short N-terminal signal peptide of HA. DNA fragments obtained from influenza seasonal and pandemic A/H1N1 reference strains were denatured and subjected to several MSSCP separations under different gel temperature profiles during the electrophoresis. As the result, the optimum MSSCP electrophoretic conditions (15 to 10 to 5°C) in which electrophoretic patterns of ssDNA fragments from seasonal and pandemic A/H1N1 strains were easily distinguishable were chosen (Fig. 1).

These optimal MSSCP separation thermal conditions were applied for the analysis of specimens collected directly from patients confirmed by real-time RT-PCR to have pandemic A/H1N1/v virus infections. Twenty-three such samples selected from a collection of travelers abroad and from domestic patients from different geographical regions of Poland who had not reported travel abroad for 1 week before the collection of specimens (Table 1) were analyzed. In four samples, we have found additional MSSCP bands which could be attributed to coinfection with a seasonal A/H1N1 strain (Fig. 2). Sample 8 predominantly had the seasonal strain MSSCP profile, with only traces of bands originating from pandemic A/H1N1/v. Either the very faint bands above the three main bands of A/H1N1/v in the case of samples 6 and 7 may be due to coinfection with pandemic/seasonal variants (other than the Brisbane seasonal variant representing the minor component), or alternatively, the minor bands could originate from a mutated A/H1N1/v strain. Although the second possibility is less likely, it should be noted that these two samples were taken from subjects infected by the virus species which originated from a Canadian visitor to Poland.

The hemagglutinin gene fragments amplified by RT-PCR from the four isolates suspected to represent two different viral variants, as shown in Fig. 2 (samples 8, 14, 15, and 19), were first sequenced directly by the Sanger DNA sequencing method. At several nucleotide positions, more than one peak on the histogram could be observed (Fig. 3A), which might reflect the presence of minor genetic variants in those samples. To verify our assumption and to identify possible minor genetic variants, the MSSCP-based minor genetic variant enrichment procedure was performed on those samples. Two electrophoretically separated bands representing putative pandemic and seasonal strains (Fig. 3) were cut out from the MSSCP gel, ssDNA fragments were recovered from those gel pieces, and the DNA was sequenced. The obtained sequences revealed clear sequencing histograms (no double peaks) and confirmed

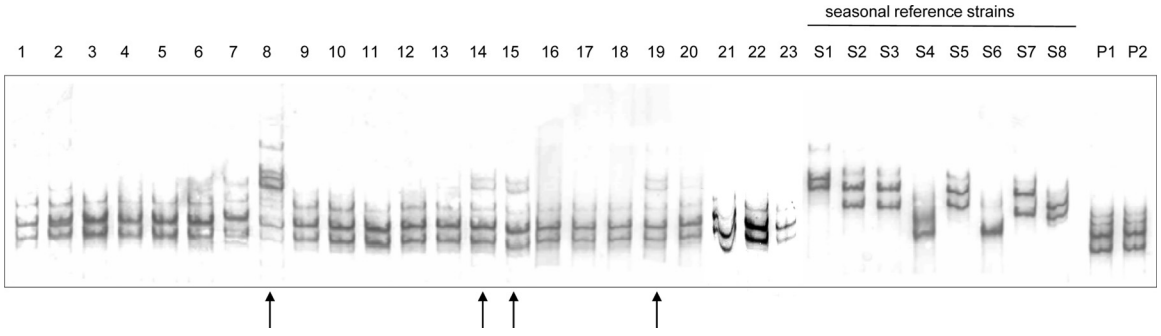

FIG. 2. Simultaneous detection of seasonal and pandemic A/H1N1 strains in specimens collected in 2009. Products of hemagglutinin gene amplification obtained for pandemic A/H1N1/v and reference seasonal strains of influenza virus A/H1N1 were denatured, and ssDNA was separated in a 9% polyacrylamide gel using the MSSCP method under optimal electrophoretic conditions. DNA bands were visualized with silver stain. The presence of both pandemic and seasonal H1N1 virus strains in four samples indicated coinfection (marked with arrows). The following lanes contain the indicated clinical specimen (sample number): 1, United States visitor (110); 2, Canada visitor (193); 3, contact with Canada visitor (195) (2); 4, Canada visitor (244); 5, United Kingdom visitor (253); 6, contact with Canada visitor (256) (4); 7, Canada visitor and contact with Canada visitor (272) (4); 8, Spain visitor (360); 9, without symptoms (383); 10, without symptoms (384); 11, Spain visitor (415); 12, Holland visitor (462); 13, India visitor (667); 14, Mexico and United States visitor (682); 15, northeast Poland (692); 16, postmortem sample, central Poland (702); 17, northwest Poland (779); 18, central Poland (751); 19, east Poland (723); 20, southwest Poland (911); 21, central Poland (837); 22, southeast Poland (888); and 23, north Poland (736). Seasonal (S) reference strains were A/Brisbane/59/2007 (lane S1), A/Fukushima/141/2006 (lane S2), A/Fukushima/97/2006 (lane S3), A/Hong Kong/2652/2006 (lane S4), A/New Caledonia/20/1999 (lane S5), A/St. Petersburg/10/2007 (lane S6), A/Solomon Islands/3/2006 (lane S7), and A/Taiwan/1/1986 (lane S8). Pandemic (P) viruses were A/Gdansk/037/2009 (H1N1) (lane P1) and A/Gdansk/036/2009 (H1N1) (lane P2).

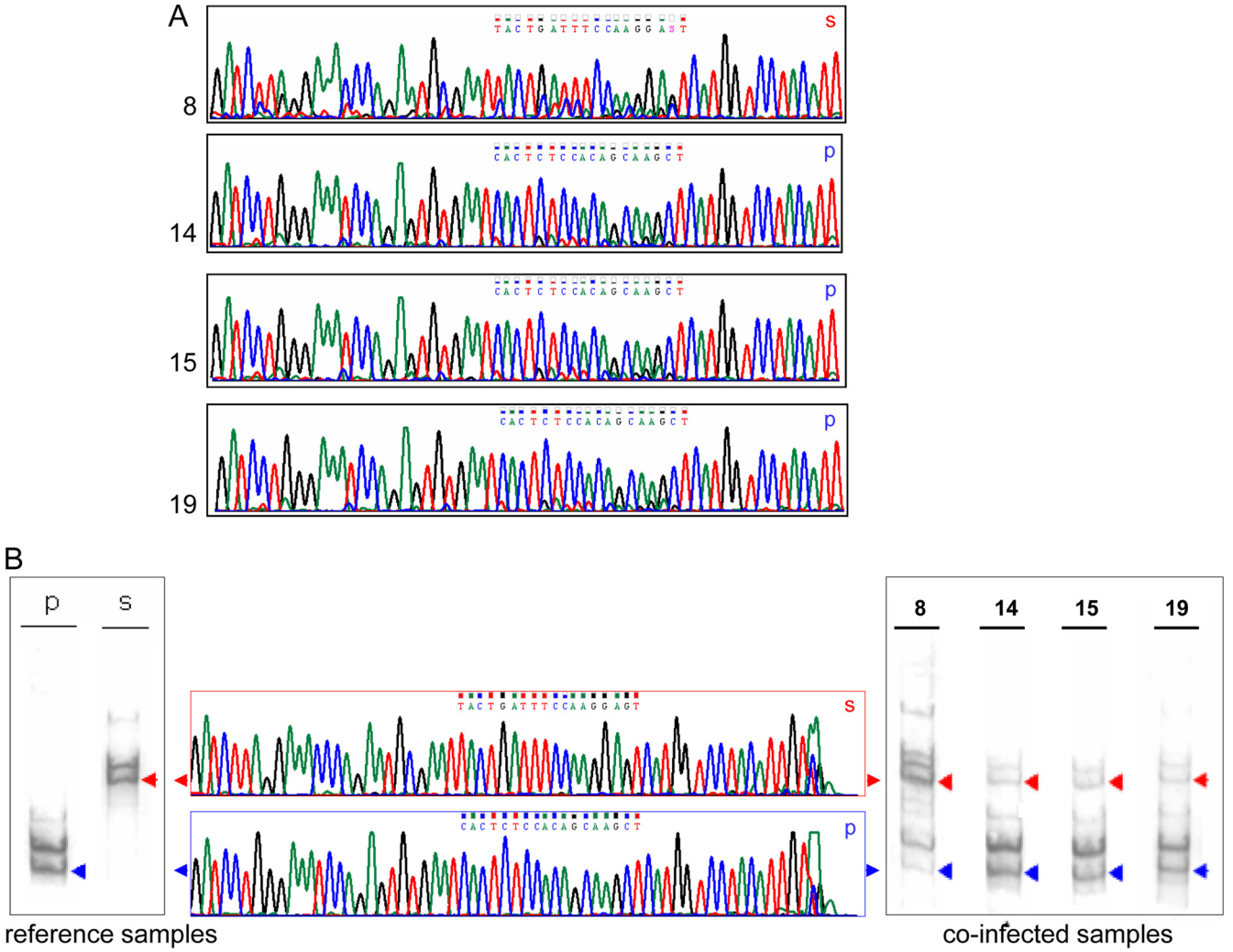

FIG. 3. Identification of minor genetic variants of A/H1N1 strains by MSSCP method and sequencing. (A) DNA sequence reads of samples found to be coinfected after direct Sanger sequencing (samples 8, 14, 15, and 19) prior to MSSCP analysis. DNA sequences correspond to sample 8 (predominantly seasonal strain) and samples 14, 15, and 19 (predominantly pandemic strains). (B) Sanger sequencing of isolates from four coinfected samples (samples 8, 14, 15, and 19) after MSSCP-based minor variant enrichment. PCR products were denatured and separated in a 9% polyacrylamide gel using the MSSCP method under optimal electrophoretic conditions. DNA bands were visualized with silver stain. The ssDNA bands indicated by arrowheads, representing seasonal (s; red) and pandemic (p; blue) strains, were cut out from the gel, and DNA was recovered and sequenced. DNA sequencing identified the presence of both seasonal (red) and pandemic (blue) isolate sequences, proving the presence of coinfection not detected by direct sequencing in all analyzed samples. Strain-specific regions are indicated by letters above the histograms.

the distinct presence of both seasonal and pandemic influenza virus variants in the four analyzed samples (Fig. 3B).

We also estimated the sensitivity of the minor genetic variant detection procedure based on the MSSCP separation. As shown in Fig. 4, RT-PCR products of the hemagglutinin gene region from seasonal and pandemic variants were mixed at a range of proportions of 50% and 50% down to 0.1% and 99.9% and analyzed. When the mixtures were analyzed by direct sequencing, the pandemic viral variant presence was detected only for the 50%/50% ratio of seasonal and pandemic variants. On the other hand, when the MSSCP sample enrichment procedure was applied, in all the analyzed mixtures, total DNA of the minor variant pandemic viral strain could be detected when it was present down to a level of 0.1% in the sample.

DISCUSSION

Several recent studies have shown that double influenza virus infection with pandemic and seasonal A/H1N1 or A/H3N2 strains may occur under natural conditions (4, 6, 8, 9). Liu et al. (9) used an RT-PCR assay followed by sequencing analysis to test 40 laboratory-confirmed cases of influenza A virus infection. Six patients were coinfected with the pandemic A/H1N1/v and seasonal A/H3N2 viruses. The authors did not observe any crucial differences in the nucleotide sequences of

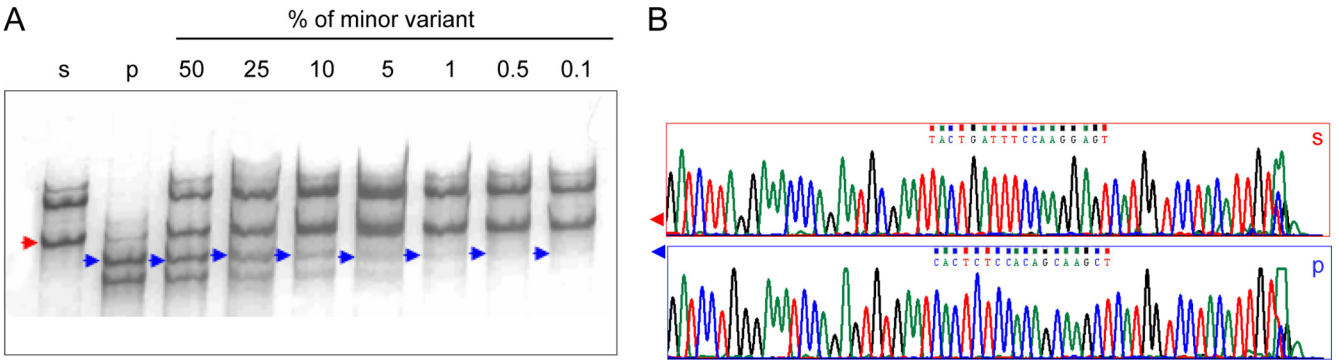

FIG. 4. Sensitivity of MSSCP identification of double A/H1N1 infection. (A) To evaluate the minimum coinfection detection level, PCR products of seasonal (s) and pandemic (p) genotypes were mixed in proportions ranging from 50% seasonal to 50% pandemic to 99.9% seasonal to 0.1% pandemic. All samples were denatured and resolved under optimal electrophoretic conditions using the MSSCP method. DNA bands were visualized by silver stain. ssDNA bands indicated by arrowheads, representing seasonal (red) and pandemic (blue) strains, were cut out from the gel, and DNA was recovered and sequenced. (B) Fragments of DNA sequence reads representing seasonal (red) and pandemic (blue) A/H1N1 strains obtained from ssDNA bands. Strain-specific regions are indicated by letters above the histograms.

pandemic and seasonal strains between patients with a dual infection and those with a single infection. Ducatez et al. (4) reported the first case of coinfection with the pandemic and seasonal A/H1N1 strains during the 2009 season in New Zealand. The authors designed a genotyping RT-PCR assay for the respective viral gene segments capable of differentiating between the seasonal and pandemic viruses. Sequencing of the amplicon only confirmed coinfection, but in some reactions cross-reactive bands were observed. On the other hand, a deep sequencing method allowed Ghedin et al. (6) to diagnose infections in immunosuppressed patients coinfected with 3 genetic variants from 2 phylogenetically distinct viral clades of pandemic H1N1/2009 influenza virus.

Dhiman et al. (3) used real-time PCR and subsequent melting temperature (T_m) analysis, which allowed discrimination between three influenza virus subtypes (pandemic A/H1N1/v, seasonal A/H1N1, and seasonal A/H3N2) on the basis of different and reproducible T_m ranges obtained for each subtype. However, the authors found 19 A/H1N1/v strains with a T_m outside the validated range for that virus (3). Also, in the case of specimens with a new minor genetic variant, the sequence of the minor variant cannot be obtained by that approach. When small numbers of samples are analyzed, the solution to that problem might provide the next-generation sequencing (NGS) method for use in a deep sequencing mode. For example, the deep sequencing method allowed Ghedin et al. (6) to diagnose infection in one immunosuppressed patient who was coinfected with 3 genetic variants from 2 phylogenetically distinct viral clades of pandemic H1N1/2009 influenza virus. However, the NGS minor genetic variant detection limit is 5% to 10% (6, 14), and the high cost of chemicals per sample and the need for extensive bioinformatic analysis of data make the NGS method practically not very useful for routine epidemiological studies at the moment. On the other hand, the MSSCP method described in this communication is robust and simple and can be used as a method to trace mixed infections with different variants of influenza virus strains. Furthermore, the MSSCP procedure combines analytical (screening) and preparative (minor variant detection) tasks in a single run. Together with the final DNA Sanger sequencing, the MSSCP-based genotyping procedure is cost-effective and could be applied to wide-scale epidemiological investigations.

Conclusions. Cocirculation of pandemic A/H1N1/v and seasonal A/H1N1 strains in the 2009-2010 epidemic season led to dual infections in patients in Poland. The MSSCP-based method of minor variant detection and genotyping of influenza A/H1N1 viruses allows not only identification of pandemic and seasonal A/H1N1 strains but also rapid and easy detection of coinfection and reassortment. To the best of our knowledge, the present report is one of the first reports of mixed infection with pandemic and seasonal A/H1N1 strains in Europe during the 2009-2010 influenza season.

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